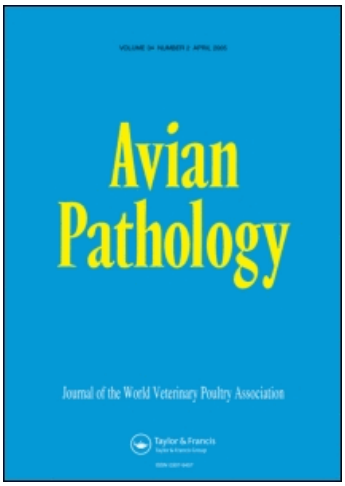

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Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poults

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Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poults

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The pathogenicity of three different type 2 turkey astroviruses (TAstV-2) was studied in specific pathogen free turkeys. These viruses differ based on sequence analysis of the capsid gene. Poults were inoculated at 2 days of age and examined during 14 days for clinical signs and virus shedding. All inoculated poults presented signs of enteric disease including diarrhoea and growth depression. Virus presence and shedding was detected by real-time reverse transcriptase-polymerase chain reaction from intestinal contents and cloacal swabs collected at 3, 7 and 14 days post-inoculation. Viraemia was also confirmed by this method. Common lesions observed at necropsy were dehydration; distended intestines filled with watery contents and undigested feed, and dilated caeca with foamy contents. Microscopic lesions present in the intestines consisted of mild crypt hyperplasia, villous atrophy and lymphocytic infiltration, and were most common in the jejunum. Presence of the viruses was demonstrated by immunohistochemistry and by *in situ* hybridization in both villi and crypt enterocytes in the jejunum and, less frequently, the duodenum, ileum and caeca. Mild lesions consisting mainly of lymphocytic infiltration were also observed in other organs including the pancreas, liver, spleen and kidneys. Mild to moderate bursal atrophy occurred in all TAstV-2-infected poults examined; however, no specific viral staining was observed in this organ or any other tissues examined apart from the intestines. In conclusion, TAstV-2 viruses with variant capsids produce a similar enteric disease in young turkeys and may also affect the immune system of the birds by causing bursal lymphoid depletion.

Introduction

Avian astroviruses belong to the genus *Avastrovirus* of the *Astroviridae* family of viruses (Index of Viruses, 2006). Astroviruses are small, non-enveloped, positive sense RNA viruses, 28 to 30 nm in diameter, and have a star-like morphology (Matsui & Greenberg, 2001). Their genome is 6.8 to 7.9 kb in length and has a 5′-untranslated region followed by three open reading frames (ORFs), a 3′-untranslated region and a poly-A tail. ORF1a and ORF1b are linked by a translational frame-shift and encode the non-structural proteins (Jiang *et al*., 1993; Gibson *et al*., 1998). Analysis of ORF1b indicates that it encodes an RNA-dependent RNA polymerase (Marczinke *et al*., 1994; Matsui *et al*., 2001). ORF2 codes for the capsid proteins (Carter & Willcocks, 1996). As with many RNA viruses, astrovirus genomes are unstable and subject to mutation and recombination (Pantin-Jackwood *et al*., 2006b). Astroviruses are linked with enteric disease in humans and young animals such as calves, lambs, pigs, dogs, cats and minks (Matsui & Greenberg, 2001). In poultry, astroviruses have been increasingly associated with enteric disease and increased mortality in young turkeys, chickens and guinea fowl (McNulty *et al*., 1980; Reynolds & Saif, 1986; Koci *et al*., 2000b; Yu *et al*., 2000b; Baxendale & Mebatsion, 2004; Guy *et al*., 2004; Cattoli *et al*., 2005), nephritis in chickens (Imada *et al.,* 2000) and a fatal hepatitis in ducklings (Gough *et al*., 1984).

Astroviruses were first found in turkeys in the United Kingdom in 1980 (McNulty *et al*., 1980), and later in the 1980s turkey astroviruses were also detected in the United States (TAstV-1) (Saif *et al*., 1985, 1990; Reynolds & Saif, 1986). A second turkey astrovirus, antigenically and genetically distinct from the previously described isolates, was later characterized (Koci *et al*., 2000b; Schultz-Cherry *et al*., 2000, 2001; Koci & Schultz-Cherry, 2002) and this and similar astroviruses have been designated type 2 turkey astroviruses (TAstV-2) (Koci & Schultz-Cherry, 2002; Cattoli *et al*., 2005; Pantin-Jackwood *et al*., 2006a,b). Astroviruses are common and were detected in 78% of turkey flocks with enteric disease in one survey (Reynolds & Saif, 1986) and have also been associated with poult enteritis mortality syndrome, a disease that has caused severe economic losses to the turkey industry (Qureshi *et al*., 2000; Barnes *et al*., 2000; Barnes & Guy, 2003). However, astroviruses have also been detected in normal/healthy turkey flocks (Reynolds *et al*., 1987; Pantin-Jackwood *et al*., 2007a). TAstV-2 was the virus most commonly found in a longitudinal survey conducted on turkey flocks, and distinct groups based on genetic sequencing were

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identified. TAstV-1 and avian nephritis virus were also found, with avian nephritis virus being detected in turkeys for the first time (Pantin-Jackwood *et al.*, 2007b).

High genetic variation has been found among TAstV-2 viruses (Cattoli *et al.*, 2005; Pantin-Jackwood *et al.*, 2006b, 2007b). These genetic changes may translate to protein changes that might have an effect on the pathogenicity and antigenicity of the viruses. Although commonly detected, not many TAstV-2 have been isolated and characterized, mostly because of the difficulty of working with these viruses. Tang & Saif (2004) reported differences in antigenicity among two TAstV-2 isolates that were 96.9% and 82.3% similar to the NC/96 TAstV-2 virus (reference strain) in their capsid gene, although it is not clear how many other serotypes of TAstV-2 exist. In addition, not many studies investigating the pathogenicity of TAstV-2 have been published and little is known about differences in disease produced by variants of the virus. TAstV-2 has been reported to affect lymphoid organs in addition to producing enteritis in turkeys (Reynolds & Saif, 1986; Thouvenelle *et al.*, 1995a,b; Qureshi *et al.*, 2000), but it is not clear whether this is associated with specific types (Tang *et al.*, 2006).

In the present study we compared the pathogenicity of three isolates of TAstV-2, which are genetically variant in the capsid genes and which represent three major phylogenetic groups (Pantin-Jackwood *et al.*, 2007b), by examining the clinical signs and gross and microscopic lesions induced by these viruses in 2-day-old specific pathogen free poults. We also determined virus spread and replication in tissues by studying the distribution of viral antigen using immunohistochemistry (IHC), and viral RNA using *in situ* hybridization (ISH).

Materials and Methods

Viruses. Three turkey astrovirus type 2 (TAstV-2) isolates were evaluated: VA/SEP-A8/03(SEP 8), TX/SEP-A313/04 (SEP 313), and CA/SEP-A270/04 (SEP 270). The viruses were isolated from the intestinal contents of commercial turkeys (Pantin-Jackwood *et al.*, 2006b). Among these TAstV-2 viruses there was an amino acid identity of between 93.8% and 97.9% in the ORF1b (polymerase gene), and an amino acid identity between 71.3% and 82.8% in the ORF2 (capsid gene) (Table 1). In addition, SEP 313 had a 2-amino-acid insertion in the ORF 2, and the SEP 8 had a 4-amino-acid insertion (Figure 1) (Pantin-Jackwood *et al.*, 2006b) (GenBank accession numbers DQ066554, DQ066572 and DQ066573). These three viruses are representative of the three most common groups of viruses, based on phylogenetic analysis of the capsid genes, found to be circulating in turkey farms in a survey conducted in 2005 (Pantin-Jackwood *et al.*, 2007b).

The viruses were isolated from 200 ml of turkey intestinal contents diluted in 1.2 ml phosphate-buffered saline (PBS), homogenized with sterile glass beads in a Fast-prep homogenizer (Thermo-Savant, Inc., Waltham, Massachusetts, USA), and centrifuged for 10 min at $800 \times g$. The supernatants were filtered serially through 0.45-μm, 0.2-μm and 0.05-μm filters and used as inocula for virus propagation in 18-day-old embryonated eggs obtained from the Southeast Poultry Research Laboratory, USDA (Athens, Georgia, USA) in-house specific pathogen free turkey flock. This flock is free of enteric viruses as indicated by periodic testing using molecular tests (Day *et al.*, 2007; Pantin-Jackwood *et al.*, 2007b); hence, the eggs and poults used here are free from enteric viruses and maternal antibodies against these viruses. Eggs were inoculated with 0.2 ml filtrates via the amniotic cavity route, and 5 days later the embryo intestines were collected, diluted 1:10 (w/v) in sterile PBS, homogenized and filtered as already described and stored at −70°C. All three viruses were passaged three times in embryos. The last passage was examined by electron microscopy to make sure no other type of virus was present in the inocula. The identity of the TAstV-2s was confirmed after each passage by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing. Also, the absence of other avian viruses (reovirus, rotavirus, coronavirus, avian nephritis virus, TAstV-1 and adenovirus) was confirmed by RT-PCR and PCR tests as previously described (Day *et al.*, 2007; Pantin-Jackwood *et al.*, 2007b). The viruses were titrated in turkey embryonated eggs (Villegas, 1998) and the titres expressed as median embryo infective doses. The lack of embryonic lesions in conjunction with negative TAstV-2 RT-PCR results on embryo intestinal contents was used to determine the endpoints.

Pathogenesis experimental design. Specific pathogen free Small Beltsville White turkeys obtained from the Southeast Poultry Research Laboratory in-house flocks were randomly divided into groups of 20 and housed in Horsfall isolators with *ad libitum* access to feed and water. Each group was inoculated with 0.2 ml appropriate virus (approximately $2 \times 10^{7.0}$ median embryo infective doses/bird) by the oral and cloacal routes (0.1 ml each route) at 2 days of age. Sham inoculated control birds were inoculated with sterile PBS. Poults were checked twice a day during the first 4 days for clinical signs of disease and daily for the rest of the experiment. At 0, 7 and 14 days post-inoculation (d.p.i.), all poults were weighed; and at 0, 3, 7, and 14 d.p.i., cloacal swabs and serum samples were collected from 10 poults and frozen at −70°C. Cloacal swabs were collected in PBS. At 3, 7 and 14 d.p.i., three poults selected at random from each group were killed by carbon dioxide exposure and examined *post mortem*. The bursa, thymus, spleen, liver, heart, kidney, proventriculus, gizzard, duodenum, pancreas, jejunum, ileum, and caecum were collected and fixed in 10% neutral buffered formalin. Sections of the intestine (jejunum, ileum, caecum) were also collected and stored at −70°C. Infection status was confirmed by testing all samples for presence of virus by RT-PCR and sequencing.

Statistical analysis. Mean body weights were analysed by one-way analysis of variance and the pair-wise multiple comparison procedure (Holm–Sidak method) (SigmaStat 3.5; Systat Software, Inc. Madison, Wisconsin, USA). The significance level was defined at $P \leq 0.05$.

RNA extraction and RT-PCR. Total RNA was extracted directly from 250 μl supernatants obtained from intestinal homogenates prepared as described above, or from 250 μl serum using Trizol LS reagent (Invitrogen Inc., Carlsbad, California, USA) according to the manufacturer's instructions. For the cloacal swab material, samples were defrosted, vortexed and centrifuged at $3 \times g$ for 10 min at 4°C prior to extraction. Extracted RNA was stored at −20°C. In order to detect the

Table 1. *Percentage amino acid identity among the TAstV-2 studied based on sequence comparisons of segments of the polymerase gene (nucleotide positions 4077 to 4880 by NC/96 numbering) and capsid genes (nucleotide positions 6208 to 7035 by NC/98 numbering) (Pantin-Jackwood et al., 2006b)*

	Polymerase gene (ORF1b)		Capsid gene (ORF2)	
	SEP 270	SEP 313	SEP 270	SEP 313
SEP 8	93.8	97.9	72.0	71.3
SEP 270		94.2		82.8

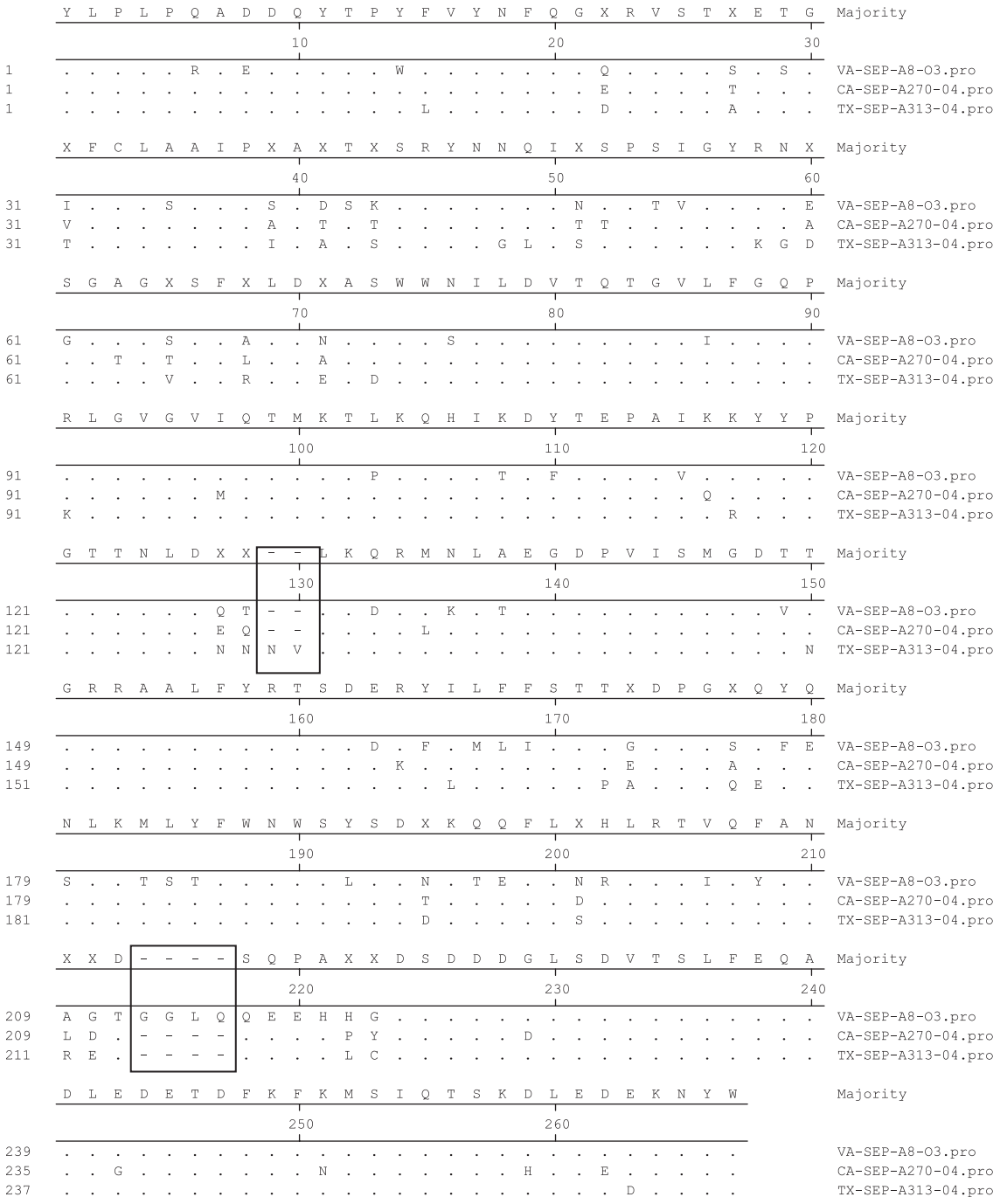

Figure 1. *Clustal W amino acid alignment of the capsid gene sequences of the three TAstV-2 viruses used in this study. (·) Positions where amino acids are identical; (—) positions where amino acids are missing. Boxed areas, amino acid insertions or deletions.*

presence of TAstV-2 in the samples mentioned, segments of the capsid gene of TAstV-2 were amplified by real-time RT-PCR as previously described (Spackman *et al.*, 2005). Briefly, real-time RT-PCR was run on a Cepheid Smart Cycler (Cepheid Inc., Sunnyvale, California, USA) in a 25 µl volume with the Qiagen OneStep RT-PCR kit (Qiagen Inc., Valencia, California, USA). Each reaction contained: 1 ×Qiagen OneStep RT-PCR kit reaction buffer, 320 mM deoxyribonucleotide triphosphate mix, 3.75 mM magnesium chloride, 10 pmol each primer, 0.1 µM TAstV-2 probe, and 1 µl Qiagen RT-PCR enzyme blend. The incubation steps consisted of 50°C for 30 min and 95°C for 15 min, followed by 35 cycles of annealing at 50°C for 30 sec, extension at 72°C for 60 sec and denaturation at 94°C for 30 sec.

To confirm the identity of the TAstV-2 capsid genotype detected, standard RT-PCR using the Qiagen one-step RT-PCR kit (Qiagen Inc.) was also done on selected samples so they could be sequenced. The set of primers used, MKCap8 and MKCap19, amplify an 849 base pair region from within the viral capsid gene of TAstV-2 (Koci *et al.*, 2000a). Amplifications were performed in a MJ Research DNA thermocycler (Waltham, Massachusetts, USA) and the incubation steps consisted of 53°C for 30 min, 95°C for 15 min and then 35 cycles of annealing at 53°C for 15 sec; extension at 72°C for 1.5 min and denaturation at 94°C for 45 sec. The RT-PCR conditions consisted of: 1 ×Qiagen OneStep RT-PCR kit reaction buffer, 320 µM each dNTP, 0.6 µM each primer and 1 µl Qiagen RT-PCR enzyme blend and 2.5 µl extracted RNA, for a total volume of 25 µl. The PCR products were separated on an agarose gel by electrophoresis and amplicons of the appropriate size were subsequently excised from the gel and extracted with the QIAquick gel extraction kit (Qiagen Inc.). Purified PCR products were then subjected to direct sequencing with the same primers used in the RT-PCR reactions, using the BigDye terminator kit (Applied Biosystems, Foster City, California, USA), run on an ABI 3730 sequencing machine (Applied Biosystems). Sequence information was analysed with the MegAling program (Lasergene 7.1; DNASTAR, Madison, Wisconsin, USA).

Histopathology. Paraffin-embedded tissues were sectioned, mounted, stained with haematoxylin and eosin, and examined by light microscopy. All samples were evaluated in a manner in which the examiner was blind to the treatment groups. Lesions were scored as follows: − = no lesions; +/− =minimal; + =mild; ++ =moderate; +++ = severe.

Immunohistochemistry. A peroxidase IHC technique using convalescent sera from TAstV-2-inoculated turkeys was used to identifyTAstV-2-infected tissues. All procedures were done at room temperature except when noted. Tissue sections were cut (4 μm thick) from paraffin-embedded tissue samples and mounted on charged glass slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, Pennsylvania, USA). Paraffin was melted from slides (15 min at 60°C) and removed by immersion in Citrisolv (Fisher Scientific) twice for 3 min each time. Slides were then washed twice in 100% ethanol for 3 min and rehydrated by sequentially immersing the slides through graded ethanol washes for 3 min each. Endogenous peroxidase activity was blocked by immersing the slides in 0.3% hydrogen peroxide for 15 min. Antigen retrieval was performed by heating the slides in citrate buffer (Citra; Biogenex, San Ramon, California, USA), maintaining the slides at 100°C for 10 min in a microwave oven. Two blocking reactions were performed, one using Power Block for 10 min (Biogenex) and another using Protein Block (goat sera) for 2 h (Biogenex).

TAstV-2 isolate-specific turkey convalescent sera collected at 21 d.p.i. from a previous experiment were used as the primary antibody. A single antibody was not developed for this purpose because the cross-reactivity among these isolates, which are genetically variant in the gene that encodes the major virus antigen, has not been determined. Antibodies were diluted 1:100 in streptavidin peroxidase antibody diluent (Biogenex) before use. Slides were incubated with the primary antibody for 1 h.

The secondary antibody was goat anti-turkey IgG, horseradish peroxidase labelled (Southern Biotechnology Associates, Inc., Birmingham Alabama, USA), and was diluted 1:100 in antibody diluent (Biogenex). The reaction was visualized with the DAB Substrate kit for peroxidase (Zymed Laboratories Inc., San Francisco, California, USA). After IHC staining, sections were counterstained with haematoxylin, air dried, coverslipped and examined by light microscopy.

Positive controls consisted of tissue sections from TAstV-2-infected poults, in which the infection had been confirmed by RT-PCR amplification. Negative controls consisted of tissues collected from sham-inoculated poults for which the infection status was confirmed to be negative by RT-PCR. Antibody controls were also included: non-immune goat sera and turkey convalescent antiserum specific for turkey reovirus (Pantin-Jackwood *et al.*, 2007a). The intensity of staining in each section was scored as follows: − =no antigen staining; +/− = rare; + =infrequent; ++ =common; +++ =widespread staining.

***In-situ* hybridization.** Three 849 base pair probes, which targeted the capsid genes of the three TAstV-2 isolates, were produced by RT-PCR as previously described (Pantin-Jackwood *et al.*, 2007a). The probe was labelled with digoxigenin by nick translation (DIG-Nick Translation Mix; Roche, Indianapolis, Indiana, USA). Two micrograms of the RT-PCR product in a total reaction volume of 20 μl was incubated at 14°C for 2 h. Completion of labelling was verified by testing an aliquot of the reaction mix on 2.5% agarose gel electrophoresis staining with ethidium bromide. Smearing and disappearance of the 849 base pair band were used to judge the adequacy of labelling.

A commercial detection kit for ISH (Link-Label-ISH Core Kit II; Biogenex) was used according to the manufacturer's instructions. Briefly, tissue sections were cut (4 μm thick) from the same paraffin-embedded tissue samples as used for histopathology and IHC, and were mounted on charged glass slides (Superfrost/Plus; Fisher Scientific). Slides were de-paraffinized and re-hydrated as previously described, then treated with Proteinase K for 10 min. Post-fixation was carried out in 1% formaldehyde for 10 min after washing in PBS. Hybridization was performed at high stringency with 2 ng/μl denatured probe, at 37°C for 16 h in 50 μl hybridization mix under a plastic coverslip in a humidified chamber. Excess probe was removed by washing in 2 ×PBS for 10 min at room temperature and was followed by two washes in 1 ×PBS for 3 min each. After incubation with Power Block Reagent for 5 min, the sections were incubated with anti-digoxigenin antibody for 2 h at room temperature. After two separate washes with 1 ×PBS, the sections were treated with streptavidin–alkaline phosphatase conjugate for 20 min at room temperature. After two separate washes with 1 ×PBS, the signal was developed with the Fast Red staining chromogen-substrate system (DakoCytomation, Carpinteria, California, USA), which produces a red precipitate indicating hybridized molecules. Sections were counterstained with Gill haematoxylin (Fisher Scientific, Middletown, Virginia, USA). Positive and negative controls slides were from the same sources as those used for the IHC procedure. Additional negative controls were also included; that is, omission of probe or the use of a probe against turkey reovirus (Pantin-Jackwood *et al.*, 2007a). Staining was observed by examination under a light microscope.

Results

Clinical signs and gross lesions. The main clinical sign observed in the TAstV-2-inoculated poults was diarrhoea accompanied by anorexia and depression. The faeces were watery to foamy, light brown–yellow in colour. The disease presentation was similar in all TAstV-2 inoculated poults regardless of the isolate. The onset of clinical signs occurred at 1 d.p.i. and persisted throughout the experimental period. Mortality occurred in virus-inoculated poults during the first 6 days; in total, five poults inoculated with SEP 8, three poults inoculated with SEP 270 and three poults inoculated with SEP 313 died. One poult from the sham-inoculated control group also died. Mortality was associated with weakness; the poults would stop eating and drinking water, and died as a consequence of anorexia. Body weights were significantly lower in the infected groups when compared with the control group at both 7 and 14 d.p.i. (Figure 2). No significant difference in body weights was observed between the TAstV-2-inoculated poults.

At 3, 7 and 14 d.p.i., three poults per group were killed. The duodenum, jejunum and ileum were filled with watery gaseous fluid, and in some cases undigested food. The caeca were dilated with foamy light brown–yellow contents. Compared with controls, the intestines of the virus-inoculated birds were two to three times bigger in diameter. Variable hyperaemia of the intestinal tract, and thinning or loss of tone of the jejunum and ileal intestinal walls, was also observed. Most birds examined at 3 d.p.i. were dehydrated. Lesions were less evident at 14 d.p.i. Lesions of the intestinal tract induced by all three viruses were similar.

Virus detection. Virus shed by the cloacal route was detected by real-time RT-PCR in all inoculated poults when examined at 3, 7 and 14 d.p.i. regardless of the isolate. The viruses were also detected in all intestinal content samples examined. All serum samples collected at 3 and 7 d.p.i. were also positive.

Histopathology. The distribution and severity of histological lesions and astrovirus antigen and RNA staining by tissue and virus isolate are summarized in Table 2. The proventriculus, gizzard and lung, which presented no lesions, very mild lesions, or which were IHC-negative or ISH-negative in all treatment groups were excluded from the table. No lesions were observed in tissues collected from sham-inoculated poults at any time.

Mostly mild lesions were present in the intestine. Shrunken degenerate cells were present in the villi and crypt epithelium and mild crypt hyperplasia resulting in

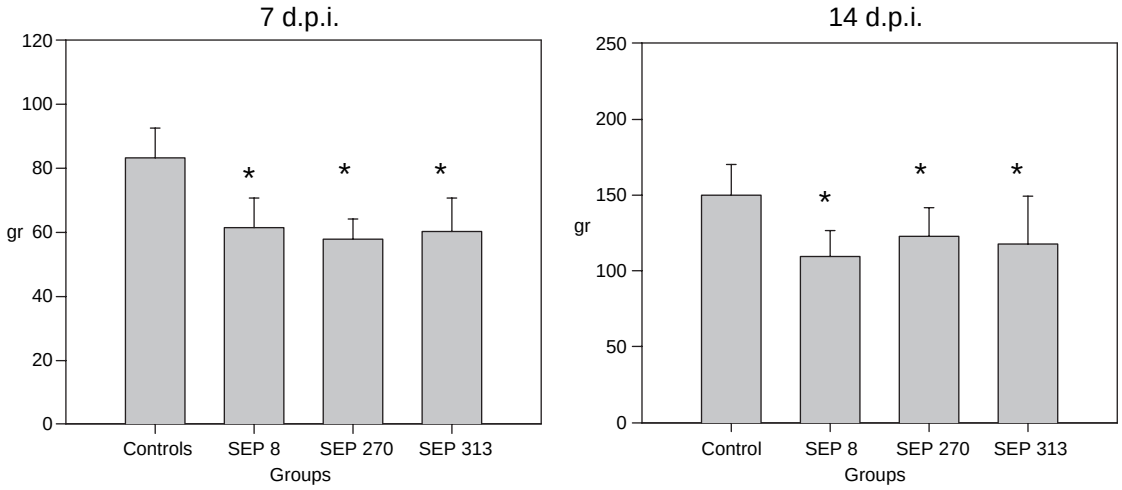

Figure 2. *Average body weights of inoculated and control poults at 7 d.p.i. and 14 d.p.i. There was a significant difference in average body weight between treated and control groups at both time points ($P \leq 0.05$).*

increased crypt depth were the most common histopathological findings (Figure 3 & 4). Mild to moderate villus shortening and increased number of lymphocytes in the lamina propria were also present and most common at 7 d.p.i. These lesions occurred principally in the jejunum. In the spleen, mild lymphoid depletion was observed at 3 d.p.i., and lymphoid follicle development was occasionally seen at 7 and 14 d.p.i. Mild multifocal randomly distributed mononuclear cell infiltrates were observed in the liver, pancreas and kidneys at 7 d.p.i. In the thymus, very mild lymphocytic depletion with an increased number of apoptotic cells was observed only at 3 d.p.i. All three viruses also induced lesions in the bursa, which were more severe at 3 and 7

Table 2. *Distribution and severity of histological lesions and viral antigen and RNA at 3, 7, and 14 d.p.i. in poults inoculated with TAstV-2 (SEP 8, SEP 270 and SEP 313)*

	SEP 8			SEP 270			SEP 313		
Tissue	Lesions[a]	IHC[b]	ISH[b]	Lesions[a]	IHC[b]	ISH[b]	Lesions[a]	IHC[b]	ISH[b]
3 d.p.i.									
Duodenum	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
Jejunum	+	++	++	+	++	++	+	++	++
Ileum	+	+	+	+	+	+	+	+	+
Caeca	+/−	+/−	+/−	+	+	+	+	+	+
Pancreas	+	−	−	−	−	−	−	−	−
Liver	+	−	−	+	−	−	−	−	
Spleen	+	−	−	+	−	−	+	−	−
Kidney	+	−	−	−	−	−	−	−	−
Thymus	+	−	−	+	−	−	+	−	−
Bursa	++	−	−	++	−	−	+	−	−
7 d.p.i.									
Duodenum	+/−	+/−	+/−	+/−	+	+/−	+/−	+/−	+/−
Jejunum	++	++	++	++	+	+	++	+	+
Ileum	+	+	+	+	+	+	+	−	−
Caeca	+/−	−	−	+/−	−	−	−	−	−
Pancreas	+	−	−	+	−	−	−	−	−
Liver	+	−	−	+	−	−	+	−	
Spleen	+	−	−	+	−	−	+	−	−
Kidney	+	−	−	+	−	−	−	−	−
Thymus	−	−	−	−	−	−	−	−	−
Bursa	+++	−	−	+++	−	−	++	−	−
14 d.p.i.									
Duodenum	+	−	−	+	−	−	+	−	−
Jejunum	++	+/−	+/−	+	+/−	+/−	++	+/−	+/−
Ileum	+/−	−	−	+/−	+/−	+/−	+/−	+/−	−
Caeca	+/−	−	−	+/−	−	−	+/−	−	−
Bursa	++	−	−	++	−	−	+	−	−

[a]Microscopic lesions: − =no lesions; +/− =minimal; + =mild; ++ =moderate; +++ =severe.
[b]Staining: − =no staining; +/− =rare; + =infrequent; ++ =common; +++ =widespread.

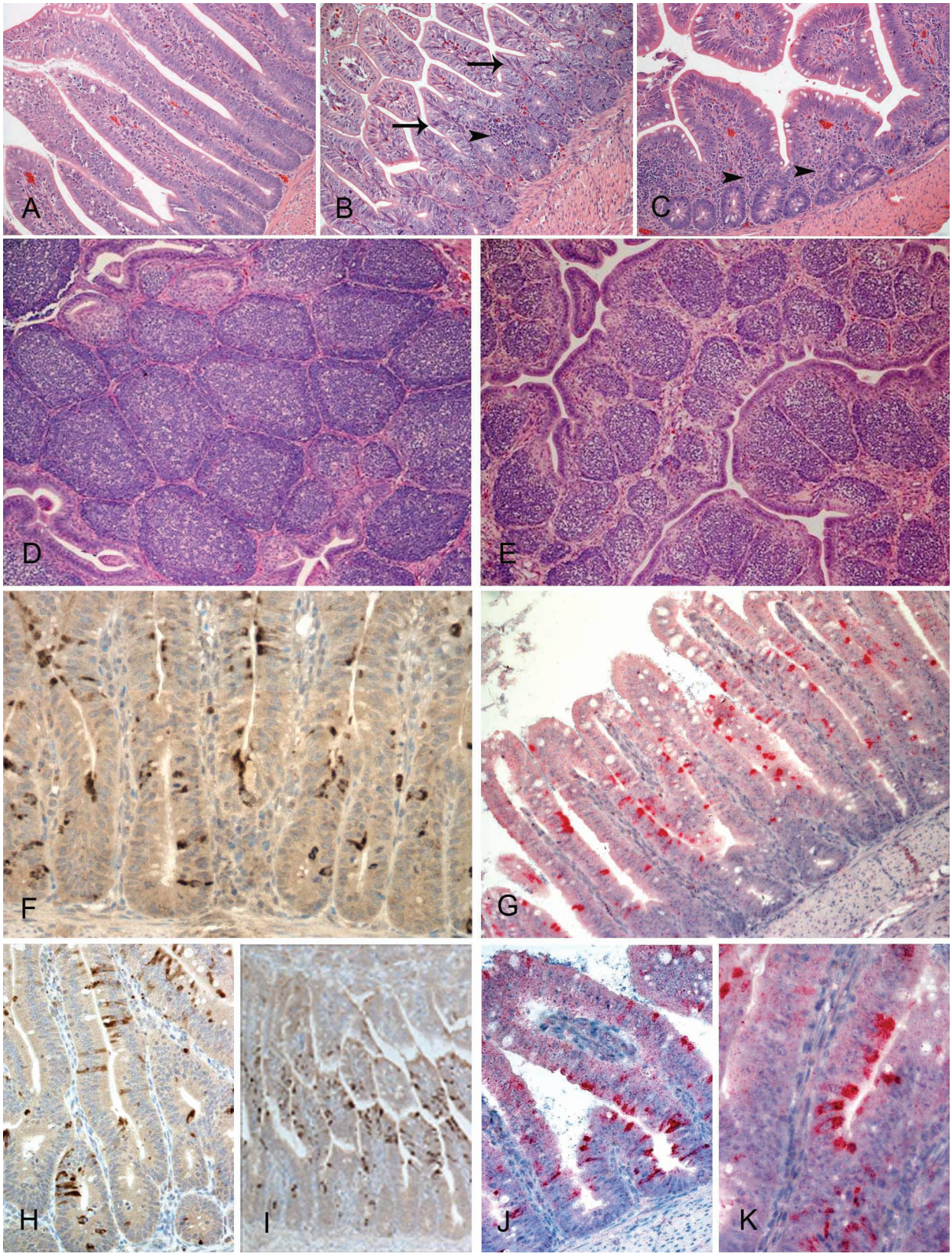

Figure 3. *Photomicrographs of poult tissue sections. 3a: Jejunum from an uninoculated poult. Haematoxylin and eosin. Magnification, 200 ×. 3b: Degenerate cells (arrows) in the epithelium of the basal section of the villi and crypt of the jejunum. There is lymphoid infiltration in the submucosa (arrowhead). Poult infected with TAstV-2 isolate SEP 270, 3 d.p.i. Haematoxylin and eosin. Magnification, 200 ×. 3c: Villi atrophy and lymphoid infiltrate (arrowheads) in the jejunum. Poult infected with TAstV-2 isolate SEP 313, 7 d.p.i. Haematoxylin and eosin. Magnification, 200 ×. 3d: Normal bursa from a sham inoculated poult, 7d.p.i. Haematoxylin and eosin. Magnification, 200 ×. 3e: Bursa with cortical and medullary follicle lymphoid depletion and stromal fibroplasia. Poult inoculated with TAstV-2 isolate SEP 270, 7d.p.i. Haematoxylin and eosin. Magnification, 200 ×. 3f, 3h, 3i: Viral antigen staining present in the cytoplasm of the enterocytes at the middle section of the affected villi (dark brown). Immunoperoxidase labelling, haematoxylin counter stain. 3g, 3j, 3k: Viral RNA staining present in the cytoplasm of the enterocytes at the middle section of the affected villi (red). Digoxigenin labelling, BCIP-NBT staining, fast red counter stain. Magnification, 200 × and 400 ×.*

d.p.i. than at 14 d.p.i., and consisted of mild to severe lymphocyte depletion from both follicle medullar and cortical zones and increased fibrous tissue between the follicles (Figure 3).

Viral antigen and nucleic acid detection in tissue. Tissues from astrovirus-infected poults, but not those from the control poults, contained detectable astrovirus antigen and RNA. Positive staining by IHC was characterized by the presence of intracytoplasmic dark-brown granules. No specific peroxidase-positive staining was seen in the tissues of the control poults. Antigen was most abundant in the jejunum (Table 2); however, antigen was also present in the duodenum, ileum and caeca. Staining was present in the cytoplasm of villus epithelial cells and less abundant in the cytoplasm of crypt epithelial cells. Positive staining was most commonly found in the enterocytes at the middle section of the affected villi (Figure 3). Apart from intestine, no other tissues had staining for TAstV-2 antigen.

Results for ISH were similar to those for IHC (Table 2). In all astrovirus-inoculated poults, positive cytoplasm staining of TAstV-2 RNA (red staining) was observed in the surface enterocytes but not in the other cells of the intestines (Table 2 and Figure 3). No ISH staining was found in the rest of the tissues examined. No virus-specific staining was detected in the controls. No differences were seen among the TAstV-2 isolates in IHC or ISH staining. The principal site of virus replication appears to be the cytoplasm of mature villous absorptive epithelial cells in the jejunum; however, viral replication also occurred in cells of the crypts and, less commonly, in epithelial cells from other sections of the intestine.

Discussion

Two-day-old turkeys inoculated with any of the three TAstV-2 viruses used in this study presented a similar enteric disease. In addition to diarrhoea, TAstV-2-infected poults had significantly lower body weights when compared with controls. At 7 d.p.i., poults inoculated with the TAstV-2 isolates were 25% lighter than controls; and they were still between 20% and 25% lighter at 14 d.p.i. Although clearly an enteropathogen, TAstV-2 induced only mild microscopic lesions in the intestines, with mild damage to the intestinal epithelium and a mild inflammatory response. Contrary to what is observed with other enteric viruses that produce enteric disease by a morphologic mechanism such as evident epithelial destruction and severe villous atrophy, or by stimulation of an inflammatory response, astroviruses appear to do so by altering the cellular physiology of the villar epithelial cells. Astrovirus infection in turkey poults causes a significant decrease in intestinal D-xylose absorption (Reynolds & Saif, 1986) and also a decrease in specific maltase activity, resulting in disaccharide maldigestion, malabsorption and subsequently osmotic diarrhoea, even though it causes only mild microscopic lesions (Thouvenelle *et al*., 1995a). This could result from enterocyte immaturity secondary to increased turnover or direct interference with disaccharidase activity (Thouvenelle *et al*., 1995b). In a recent study, it was also demonstrated that astroviruses may increase the intestinal barrier permeability, which could lead to increased fluid secretion into the intestinal lumen (Moser *et al*., 2007).

Lymphoid depletion occurring in immune organs as a consequence of astrovirus infection has been reported previously (Qureshi *et al*., 2000; Behling-Kelly *et al*., 2002; Tang *et al*., 2006). All three viruses studied induced mild to moderate lymphocyte depletion in the bursa; however, only minimal microscopic lesions were detected in the thymus and spleen of poults. No viral antigen or RNA staining was present in any of the lymphoid tissues examined, similar to what was found in a previous study (Behling-Kelly *et al*., 2002). However, low levels of viral replication, undetectable by the techniques used in this study, may still occur in the bursa (Qureshi *et al*., 2000). The lymphocyte depletion observed could be a consequence of apoptosis indirectly induced by TAstV-2 replicating in other cells of the bursa (i.e. bursal epithelial cells). This virus-independent or "by-stander" lymphoid depletion has also been identified in several other avian viral infections (Jungmann *et al*., 2001; Perkins & Swayne, 2001; Pantin-Jackwood *et al*., 2007a). The maldigestion and malabsorption observed in TAstV-2 infected poults, and consequently the nutri-

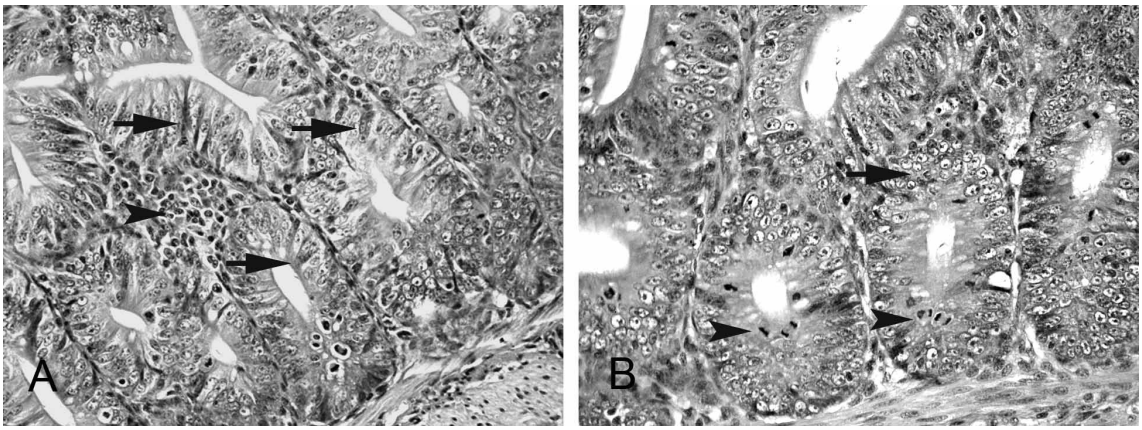

Figure 4. *Photomicrographs of poult tissue sections. 4a: Degenerate cells (arrows) in the epithelium of the villi and crypt of the jejunum. Diffuse infiltrate of lymphocytes, plasma cells and macrophages (arrowhead). Poult infected with TAstV-2 isolate SEP 270, 3 d.p.i. Haematoxylin and eosin. Magnification, 400 ×. 4b: Crypts of the jejunum increased in depth and lined by hyperplastic epithelial cells (arrow). Numerous mitotic figures in the crypt epithelium (arrowheads). Poult infected with TAstV-2 isolate SEP 313, 7 d.p.i. Haematoxylin and eosin. Magnification, 400 ×.*

tional deficiencies induced, could also play a role in the bursal atrophy observed in these birds.

In the present study, early mortality occurred in poults inoculated with the TastV-2 viruses. This has also been reported in a previous study in which it was shown that turkey astrovirus infection alone resulted in mortality as high as 11% in challenged poults (Yu *et al*., 2000a). Although still not clear, the mortality most probably occurred because of the viral challenge the poults received at a very young age, which induced a severe enough lethargy and feed and water withdrawal that the poults died. We chose to use poults this young in order to mimic field conditions, where poults are exposed to astroviruses as soon as they are placed in the houses, since astroviruses are highly stable in the environment and may remain in the litter for a long time (Yu *et al*., 2000b). In a previous study, the pathogenicity of two turkey astrovirus isolates, TAstV1987 and TAstV2001, was compared in older turkey poults (14 to 28 days old). Both viruses caused enteritis and growth depression but no mortality (Tang *et al*., 2006).

In the present study we also describe the distribution of TAstV-2 in the tissues of experimentally infected poults. IHC and ISH were used for detecting the virus and, in terms of staining intensity and tissue distribution of viral signals detected, revealed similar results. Among all the tissues examined, only the intestines were consistently positive for the presence of virus. The specific cell types infected were limited to enterocytes. The localization of peroxidase-positive brownish granules in the cytoplasm of the affected cells by IHC, and reddish granules by ISH, is consistent with the intracellular distribution of astroviruses. Similar results to those obtained here have been reported with other TAstV-2s (Behling-Kelly *et al*., 2002; Koci *et al*., 2003).

As previously reported (Koci *et al*., 2003), viraemia occurred after infection with TAstV-2; however, only intestinal cells appear to support viral replication. The possibility exists that low levels of viral replication may occur in other tissues and not be detected with the tests used. This would also explain the extra-intestinal lesions observed, such as bursal depletion and lymphocytic infiltration in organs such as the liver, pancreas, kidney and spleen.

In conclusion, the pathogenesis of the three TAstV-2s studied was very similar in spite of the substantial genetic variation found among the capsid of the viruses. Changes in the capsid may affect antigenicity, but do not appear to have an effect on disease presentation or on the extent and distribution of viral replication in tissues.

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